The transcription factor ELF4 controls quiescence of endothelial cells and their resistance to myeloablative treatments in bone marrow

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Abstract

Objective—The regeneration of the hematopoietic system in bone marrow after chemotherapy depends on a balance between the quiescence and proliferation of lineage-specific progenitor cells. Even though the vascular network in bone is damaged by cytoablation, the transcriptional control of quiescence in endothelial cells is not well known. In this study, we investigated the role of the transcription factor ELF4 in the proliferation of endothelial cells in bone marrow.

Methods and Results—Loss-of-function models were used to study the role of ELF4 in human and murine endothelial cells. ELF4 promotes cell-cycle entry by activating CDK4 in human umbilical vein endothelial cells. *Elf4*-null mice exhibited enhanced recovery of bone marrow CD45$^-$ CD31$^+$ endothelial cells and sinusoidal blood vessels following administration of 5-fluorouracil.

Conclusion—Loss of ELF4 leads to increased quiescence in bone marrow endothelial cells by the deregulation of CDK4 expression and to enhanced regeneration of sinusoidal blood vessels.

Keywords

endothelial cells; bone marrow ablation; transcription factor; ELF4; quiescence

Introduction

Cellular quiescence protects tissue-specific progenitor cells from cell-cycle dependent cytotoxicity to ensure longevity and regenerative potential. This reversible cell-cycle arrest is actively regulated by cell-intrinsic factors, in addition to proliferation-inducing signals from the milieu[1]. Chemotherapeutic drugs that target dividing cells cause ablation of hematopoietic and endothelial cells in the bone marrow (BM) [2, 3]. The molecular events that protect endothelial cells from apoptosis and disruption of cell-cell junctions following

administration of cytotoxic drugs that ultimately lead to the regression of sinusoidal blood vessels in BM are not well-understood [4].

ELF4 is a member of the ETS family of transcription factors defined by a highly conserved DNA-binding domain (ETS domain) [5, 6]. ELF4 has a tumor suppressor function in acute myelogenous leukemias by gene repression or chromosomal translocation with the ERG gene [7-10]. However, ELF4 can also have oncogenic properties in ovarian cancer [11]. In the hematopoietic system, ELF4 activates the cytotoxicity of natural killer cells and negatively regulates quiescence in hematopoietic stem cells [12, 13]. We recently reported that ELF4 also inhibits T cell proliferation downstream of TCR and mTOR signaling by activating the tumor suppressor KLF4 [14, 15]. In this work, we addressed whether ELF4 controls proliferation in a non-hematopoietic cell lineage.

ETS proteins are important transcriptional regulators for the development and function of endothelial cells [16]. Furthermore, the enhancers and promoters of many endothelial-specific genes are regulated by ETS proteins [16]. For instance, ETS1, ERG, Fli-1, ELF1 and TEL are involved in vascular development and angiogenesis [17]. ELF4 is highly homologous to ELF1, ELF2 (a.k.a. NERF2), ELF3 and ELF5 [6], a sub-family involved in the regulation of Tie2 gene expression [18, 19]. Similarly to ETS1, deletion of the *Elf4* gene is not embryonic lethal, suggesting either functional redundancy or function in adult tissues. Despite the lack of developmental defects observed in loss-of-function mouse models generated by homozygous gene deletion, they can still provide information about unique functions in processes of vascular remodeling in adult tissues not compensated by homologous ETS proteins [20-24].

The vasculature in BM is a complex network that includes sinusoidal blood vessels (SBV) that traverse the bone cavity from the endosteum to the central sinus. In contrast to other blood vessels, SBVs are formed by a discontinuous single layer of endothelial cells to allow maximal permeability [25]. Thus, hematopoietic cells and metastatic tumor cells (i.e. breast and prostate cancer) can exit or home in BM via SBVs. An additional function of BM endothelial cells is to provide HSCs with a microenvironment that signals proliferation, differentiation, and mobilization [2, 26, 27]. Chemically induced cytoablation leads to a collapse and regression of SBVs due to a reduction of BM cellularity that normally supports the vascular network in BM [2, 3, 28]. The regulation of quiescence in endothelial cells during homeostasis has not been previously addressed. We postulated that quiescent endothelial cells in BM would be spared from chemical ablation, as happens with HSCs, resulting in heightened regeneration of blood vessels in BM.

In this work, we report that ELF4 promotes cell-cycle entry in human umbilical-cord endothelial cells (HUVEC). Thus, silencing of ELF4 gene expression led to increased quiescence in HUVEC cells due to the downregulation of CDK4 expression. In agreement with this finding, *Elf4*$^{-/-}$ mice revealed enhanced recovery of BM CD45$^-$ CD31$^+$ endothelial cells and regeneration of sinusoidal blood vessels after cytoablation, due to a lower proliferation rate at steady state. These data support the hypothesis that ELF4 negatively regulates quiescence in endothelial cells similarly to hematopoietic stem cells[13].

Methods

Cells

Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza and cultured following the manufacturer's instructions. NIH3T3 and COS-7 cells were obtained from ATCC.

Mice

Elf4⁻/⁻ mice were obtained from S. Nimer and described previously [12]. C57BL/6 mice were purchased from Jackson Laboratories. For cytoablation, a single dose of 5-fluorouracil (5-FU) was administered intraperitoneally (150 mg/kg). All mice were bred and maintained in specific pathogen-free conditions at the Baylor College of Medicine. All experiments were approved by the Institutional Animal Care and Usage Committee of Baylor College of Medicine.

Promoter assays

ELF4 cDNA was cloned into the CMV5 expression vector. cMYC cDNA was obtained from Addgene and then cloned into the CMV5 plasmid. The human CDK4 promoter obtained from Addgene was cloned into the pGL3-basic vector (Promega). COS-7 and NIH3T3 cells were transfected with a mixture of plasmids (CMV5-ELF4 or CMV5-cMYC), pGL3-CDK4 and renilla luciferase plasmids using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured using the dual-luciferase reporter assay system (Promega) and a luminometer (Sirius, Berthold).

Detection of ELF4 expression

HUVEC cells were grown on slides and fixed with 1% paraformaldehyde in PBS for 10 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 minutes and then incubated with 5% bovine serum albumin in PBS for 30 minutes. HUVEC cells were incubated with rabbit anti-human ELF4 (Orbigen) followed by anti-rabbit IgG conjugated with Alexa 488 (Invitrogen). Phalloidin-Alexa 555 (Invitrogen) was used to detect F-actin. Murine endothelial cells were purified from BM using anti-CD31 and BD-IMag (BD Biosciences) and spun onto glass slides. Following fixation and permeabilization as described above, cells were incubated with anti-CD45-FITC or anti-CD31- FITC, and anti-ELF4[12]. Slides were mounted with ProLong Gold antifade reagent (Invitrogen), and images were taken using a NIKON 90i microscope. ELF4 expression was also determined in paraffin fixed sections of mouse embryos (14.5 d.p.c.) using an affinity purified polyclonal antibody against ELF4 as previously described[13].

Cell cycle analysis

DNA content was determined using propidium iodine as described previously[13] and analyzed using a FACScanto cytometer (BD Bioscience) and ModFit LT software (Verity). For the nuclear detection of Ki67, cells were fixed and permeabilized using reagents from the apo-BrdU kit (BD Bioscience) and stained with anti-Ki67-FITC (Vector Laboratories) and 7-AAD. Murine endothelial cells were identified by flow cytometry using anti-CD31 and anti-CD45 antibodies. Samples were analyzed using FACScanto cytometer and FlowJo software (Tree Star).

Levels of cell-cycle regulators in HUVEC cells treated with control shRNA or ELF4shRNA were evaluated by immunoblots using polyclonal anti-p21 (eBioscience) and rabbit polyclonal anti-p27, mouse anti-cyclin D1, mouse anti-cyclin D3, mouse anti-cdk4, and rabbit anti-phospho-Rb (Cell Signaling Technology). ELF4 was detected using rabbit polyclonal anti-ELF4. β-actin was used to normalize sample loading.

Detection of blood vessels

Femurs were fixed and decalcified (Fisher Diagnostic) and embedded in paraffin. To identify bone vasculature, sections were stained with anti-MECA32 (Becton Dickinson). Morphometric analysis was performed to enumerate capillaries and sinusoidal blood vessels in different sections using NEI-Elements software (Nikon). Capillaries and sinusoidal blood

vessels were identified based on morphology (hematoxylin and eosin staining) and MECA32 expression [2, 3]. Tissue sections were reviewed by a pathologist at Texas Children's Hospital. BM cells were cultured on fibronectin-coated plates for 14 days in F12K media (ATCC) supplemented with 10% FBS, 0.1 mg/ml heparin and 0.03 mg/ml of endothelial cell growth supplement (ECGS). Adherent cells were incubated with Alexa Fluor 488 Dil-ac-LDL (5 ug/ml) for 3-4 hours at room temperature and positive colonies were counted under fluorescence microscope. Percentage of Dil-ac-LDL positive endothelial cells was confirmed by flow cytometry using F4/80 antibody to rule out macrophages.

Immunofluorescence in yolk sac

Mouse embryos (9.5 d.p.c.) were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, and blocked with MOM solution (Vector Laboratories). Yolk sac was stained overnight with mouse anti-smooth-muscle actin (DAKO), rat anti-CD31 (Beckton Dickinson) and affinity purified rabbit anti-ELF4 at 4 °C. Tissues were then incubated with anti-mouse Alexa 488, anti-rat Alexa 633 and anti-rabbit Alexa 568 (Invitrogen). Images were taken using a Zeiss Axioplan 2 microscope.

Gene silencing in HUVEC cells

HUVEC cells were transfected with 30 pmol ELF4 Stealth RNAi or Stealth RNAi negative control (medium GC content) using lipofectamine RNAiMax (Invitrogen) for 6 hours at 37 °C. Cells were cultured for an additional 48 hours in EGM-2 medium. Expression of ELF4 was measured by real-time PCR using the following primers: 5'-TCCGAAATGC TTCCAGACTC-3' and 5'-GGGTCAGTGACAGGTGAGGT-3' and for βactin: 5'-CAAACA TGATCTGGGTCATCTTCTC-3' and 5'-GCTCGTCGTCGA C AACGGCTC-3'

Results

Loss of ELF4 leads to enhanced regeneration of sinusoidal blood vessels in bone marrow

We previously described ELF4 as a negative regulator of quiescence in HSC during homeostasis but not during regenerative hematopoiesis [13]. The increased hematological recovery post cytoablative treatment observed in $Elf4^{-/-}$ mice was due to a larger pool of more quiescent HSCs [13]. Based on this finding, we tested whether ELF4 also controls neovascularization of BM by regulating quiescence of endothelial progenitor cells. We first confirmed that ELF4 is expressed in the nuclei of HUVEC cells, endothelium of blood vessels and endocardium of 14.5 d.p.c. mouse embryos (Figure 1A, B). To correlate ELF4 expression with blood vessels, we performed whole-mount immunofluorescence of the yolk sac of 9.5 d.p.c. embryos using CD31 as marker of endothelial cells (Figure 1C). Finally, we purified CD31+ murine BM cells and observed that ELF4 is expressed in both hematopoietic (CD45 positive) and non-hematopoietic (CD45 negative) cells (Figure 1D). ELF4 expression showed a nuclear punctuate pattern in $CD45^-$ $CD31^+$ BM cells, which was reminiscent of the expression pattern in HSC [13], suggesting that ELF4 is not associated with any well-defined nuclear domain. Collectively, these findings confirm that ELF4 is expressed in endothelial cells.

Both hematopoietic and vasculature systems are disrupted in the BM after administration of 5-FU [3]. Because ELF4 promotes cell-cycle entry of HSCs at steady state [13], we monitored the expansion of BM endothelial cells and regeneration of sinusoidal blood vessels in ablated $Elf4$-null mice. We enumerated $CD45^-$ $CD31^+$ BM cells in femurs of 5-FU-treated mice by flow cytometry (Figure 2). Increased expansion of BM $CD45^-$ $CD31^+$ endothelial cells was observed in $Elf4^{-/-}$ mice between 9 and 12 days. $Elf4^{-/-}$ femurs also exhibited increased numbers of endothelial cells 3 days after 5-FU administration, indicating that more

endothelial cells survived this treatment compared to wild type mice. We confirmed this acute effect by culturing BM cells on fibronectin-coated plates and incubating them with Dil-Ac-LDL to count colonies of endothelial cells representative of the initial number in BM isolate. Three days after 5-FU administration, the number of Dil-Ac-LDL-positive colonies recovered from BM was significantly higher in $Elf4^{-/-}$ femurs compared to wild type femurs (Figure 3A).

To investigate whether this increase in number of BM endothelial cells leads to heightened regeneration of sinusoidal blood vessels (SBVs), we examined the expression of MECA-32, a pan-endothelial marker, in femurs of wild type and $Elf4^{-/-}$ mice after 5-FU treatment (Supplemental Figure 1). Even though the number of capillaries in $Elf4^{-/-}$ femurs was similar to wild type femurs, the number of SBVs was significantly higher in $Elf4^{-/-}$ femurs, correlating with a peak of expansion in $CD45^-$ $CD31^+$ BM cells (Figure 3B). This finding suggested increased quiescence of cells at steady state and/or enhanced regenerative proliferation of $Elf4^{-/-}$ $CD45^-$ $CD31^+$ BM cells. Even though the enumeration of BM endothelial cells by flow cytometry has limitations, $Elf4^{-/-}$ mice showed increased numbers of $CD45^-$ $CD31^+$ cells at the nadir (Figure 2, $9,498\pm2,497$ in +/+ versus $15,076\pm3,362$ in −/ −, n = 5) consistent with a higher number of Dil-ac-LDL-positive colonies (Figure 3A). Therefore, a paucity of proliferation in $Elf4^{-/-}$ BM endothelial cells could account for the improved regeneration of BM vasculature.

ELF4 regulates proliferation in bone marrow endothelial cells

We previously reported that ELF4 promotes cell-cycle entry in HSCs and induces cell cycle arrest in naïve $CD8^+$ T cells [13, 14]. These findings suggest that ELF4 activates the expression of genes involved in cell cycle regulation in a cell context-dependent manner. Therefore, we investigated whether ELF4 also controls the proliferation of BM endothelial cells during homeostasis. Analysis of DNA content revealed a lower percentage of $Elf4^{-/-}$ $CD45^-$ $CD31^+$ BM cells in S-phase during homeostasis (Figure 4A, B). Next, a combination of Ki67 expression in nuclei and DNA content was used to distinguish cells in G_0 from G1. $Elf4^{-/-}$ $CD45^-$ $CD31^+$ BM cells showed a significant increase in G_0 phase ($Ki67^{-/lo}$ 2n DNA) of the cell cycle (Figure 4C,D). This finding suggests that a relatively low proliferative capacity of $Elf4^{-/-}$ BM endothelial cells, defined as $CD45^-$ $CD31^+$, leads to accelerated survival and regeneration of SBVs, coinciding with the hematological recovery previously reported [13]. Notably, we did not detect differences in the proliferative capacity of BM endothelial cells after 5-FU administration (not shown).

We next investigated the effects of ectopic expression of ELF4 in the proliferation of HUVEC cells using a fusion protein. Cells were nucleofected with an ELF4-GFP expression plasmid, purified by cell sorting and analyzed by DNA content. ELF4 induced the proliferation of HUVEC cells, as shown by a higher proportion of cells in S-phase (Figure 5A). Conversely, silencing ELF4 in HUVEC cells using gene-specific shRNA led to a significant reduction of the number of cells in S-phase (Figure 5B). To further discriminate cells in G_0 and G_1 and determine whether quiescence was specifically altered by ELF4, we measured the expression of Ki67 in conjunction with DNA content. As shown for BM cells, quiescent HUVEC cells were defined as cells with 2n DNA content that were negative for Ki67. Loss of ELF4 resulted in a significant increase in the percentage of cells in G_0, suggesting that ELF4 promotes cell cycle entry of endothelial cells (Figure 5C, D). In contrast to ELF4, silencing of ELF1 did not significantly affect the percentage of 2n $Ki67^-$ cells (Figure 5C, D). The difference in the magnitude of quiescence between primary murine $CD45^-$ $CD31^+$ and HUVEC cells is likely due to cellular heterogeneity and the fact that commercial HUVEC cells are pre-selected for proliferation. Nevertheless, it is clear in both systems that loss of ELF4 reduces proliferation of endothelial cells and increases the

proportion of cells in G_0. In addition to regulating proliferation of HUVEC cells, ELF4 modulates tube formation and wound healing (Supplemental Figure 2).

The use of HUVEC cells allowed us to further investigate the mechanism of cell cycle regulation by immunoblot analysis. As expected from the proliferation assays, loss of ELF4 activity resulted in decreased phosphorylation of Rb (Figure 6A). In contrast to normal levels of Cyclin D1, Cyclin D3, and p21, the expression of CDK4 was diminished by 40% (Figure 6A). A rather discrete reduction in CDK4 levels may be due to a combination of transient ELF4 mRNA silencing and the half-life of CDK4 protein. Reduced proliferation of HUVEC cells treated with ELF4 shRNA could be due to lower expression of CDK4. We performed promoter assays to confirm that ELF4 directly regulates expression of the CDK4 gene. We found that ELF4 activates the CDK4 promoter as efficiently as c-MYC, a known activator of CDK4 (Figure 6B). Collectively, our data suggest that ELF4 can promote cell-cycle entry of endothelial cells, at least in part by regulating CDK4 expression.

Discussion

Quiescence is a reversible cell cycle arrest that prevents differentiation and ensures longevity of lineage-specific progenitor cells. A better understanding of the regulation of quiescence in endothelial cells is necessary to minimize vascular damage and to accelerate neovascularization of BM in patients undergoing chemo- or radiation therapies. In this study, we demonstrated that the transcription factor ELF4 negatively regulates quiescence in human umbilical cord endothelial cells *in vitro* and in murine BM endothelial cells *in vivo*.

The absence of a vascular phenotype in *Elf4*^{−/−} mice suggests that this transcription factor may have a functional role in vascular remodeling of adult tissues rather than during embryogenesis. Therefore, we tested whether loss of the *Elf4* gene leads to alterations in the vascular regeneration post-myelosuppression induced by 5-FU administration [2, 3]. *Elf4*^{−/−} mice showed enhanced recovery of CD45^{−} CD31^{+} endothelial cells and sinusoidal blood vessels peaking approximately two weeks after cytoablation, consistent with the kinetics of blood vessel recovery previously reported [3]. Even though Tie2 signaling is essential for the remodeling and maturation of new vessels and maintenance of a 'resting state' in established blood vessels [29, 30], *Elf4*^{−/−} mice crossed to *Tie2*-LacZ transgenic mice did not show significant reduction in Tie2 expression in different tissues (not shown). We hypothesized that the enhanced restoration of sinusoidal blood vessels in the BM of *Elf4*^{−/−} mice was due to cell cycle regulation in BM endothelial cells at steady state.

A limitation in our study is the lack of well-defined cell surface markers to identify BM endothelial progenitor cells by flow cytometry. However, the use of human derived endothelial cells allowed us to study the role of ELF4 in proliferation with greater detail. Interestingly, loss of ELF4 function resulted in increased quiescence of HUVEC cells, reduced phosphorylation of Rb, and lower expression of cyclin-dependent kinase 4 (CDK4). There is emerging evidence that CDK4 activity is involved in cellular quiescence. Inhibition of CDK4/6 activity has been shown to increase resistance to radiation in hematopoietic cells [31]. GATA-2 induces HSC quiescence by reducing the levels of CCND3, CDK4 and CDK6 [32]. In skin stem cells, NFATc1 targets CDK4 to control stem cell quiescence [33]. In addition to proliferation, transient silencing of ELF4 in HUVEC cells affected their ability to heal a wound and to form tube-like structures in Matrigel (Supplemental Figure 2). However, ELF4 silencing in HUVEC cells did not alter cell-cell contacts in a monolayer or VE-cadherin levels (not shown). Our findings uncovered a new role of ELF4 in endothelial cells: control of quiescence by activating expression of CDK4.

Our study supports the model that a heightened recovery of sinusoidal blood vessels in $Elf4^{-/-}$ mice is associated with the deregulated proliferation of endothelial cells. The paucity of endothelial cells to proliferate in the absence of ELF4 can explain the increased resistance to cell cycle-dependent toxicity because endothelial cells in G_0 phase can escape the deleterious effect of 5-FU. Consequently, more "progenitor" endothelial cells can rapidly regenerate sinusoidal blood vessels in $Elf4^{-/-}$ mice. Collectively, we propose that ELF4 has dual functions in hematological recovery post-BM ablation by regulating cell cycle entry in both hematopoietic stem cells [13] and in BM endothelial cells. As a consequence, ELF4 activity can contribute to the regeneration of a functional vascular niche. Gene modulation of the ELF4 gene could be used in the future to enhance hematological recovery of patients undergoing chemotherapy or radiation by accelerating the regeneration of both the hematopoietic system and the vascular niche. The determination of the genes regulated by ELF4 in BM endothelial cells and the mechanism by which ELF4 is regulated remain to be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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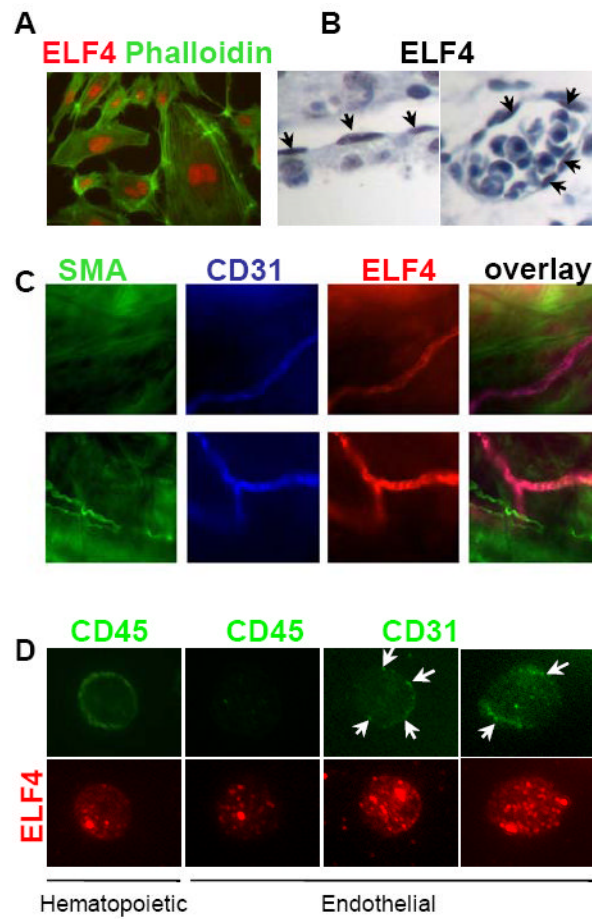
Figure 1. The transcription factor ELF4 is expressed in endothelial cells
(A) Nuclear expression of ELF4 was determined by immunofluorescence in HUVEC cells using anti-ELF4 (red) and phalloidin (green). Magnification: 40x. (B) Immunohistochemistry in E14.5 embryos using an anti-ELF4 antibody. Arrows indicate positive endothelial cells in endocardium (left) and a blood vessel (right). Magnification: 20x. (C) Immunofluorescence of yolk sac (E9.4) using smooth muscle actin (SMA, green), anti-CD31 (blue), and anti-ELF4 (red). (D) Immunofluorescence of murine hematopoietic (CD45+) and non-hematopoietic endothelial cells (CD45− CD31+). Arrows indicate CD31 surface expression. Magnification: 40x.
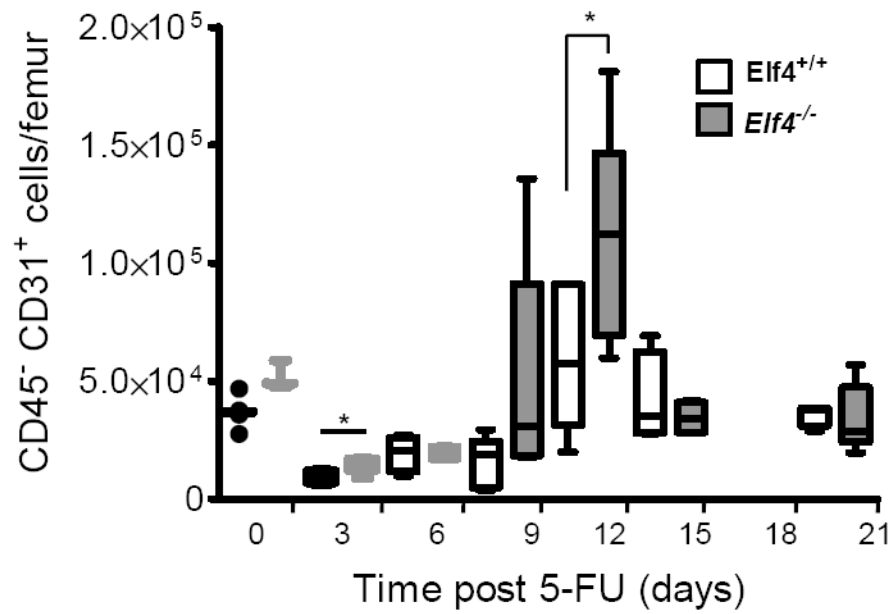
Figure 2. Enhanced recovery of endothelial cells in BM of *Elf4⁻/⁻* mice
The number of CD45⁻ CD31⁺ cells after administration with a single dose of 5-FU was analyzed by flow cytometry in wild type and *Elf4⁻/⁻* femurs and represented in box plots (5 mice per time point). The solid horizontal lines represent median values (Two-tailed Student's t-test, * P< 0.05).
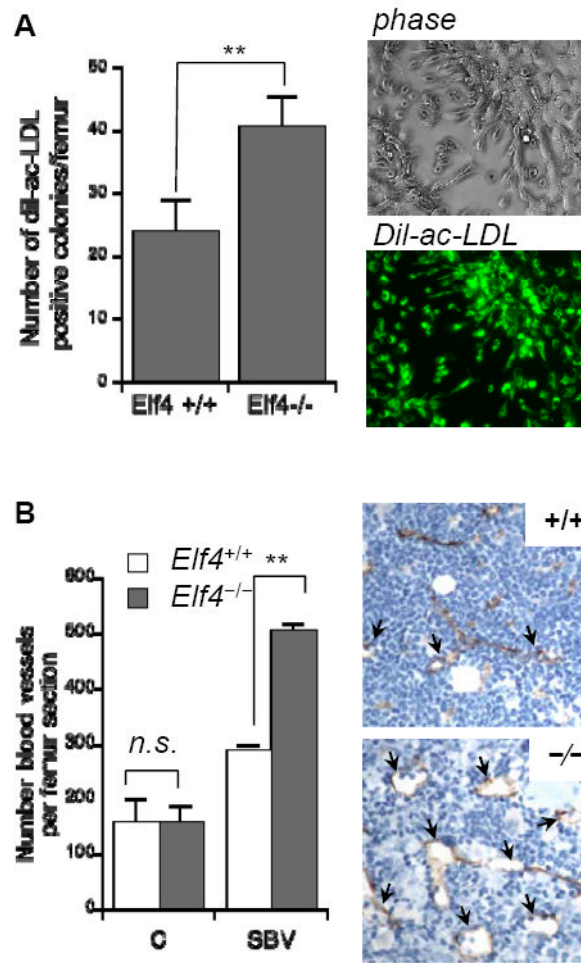
Figure 3. Loss of ELF4 leads to increased numbers of sinusoidal blood vessels in BM
(A) BM cells from wild type and *Elf4*$^{-/-}$ mice were collected after three days of 5-FU administration, cultured for two weeks on fibronectin-coated plates and incubated with Alexa Fluor 488 Dil-Ac-LDL. The number of Dil-ac-LDL-positive colonies is shown on the left, and a representative colony is shown on the right. (B) The pan-endothelial marker MECA32 was used to identify capillaries (C) and sinusoidal blood vessels (SBV) in femurs of mice treated with 5-FU 14 days earlier. Arrows indicate representative SBV. Two-tailed Student's t-test, n = 3, ** P< 0.01.
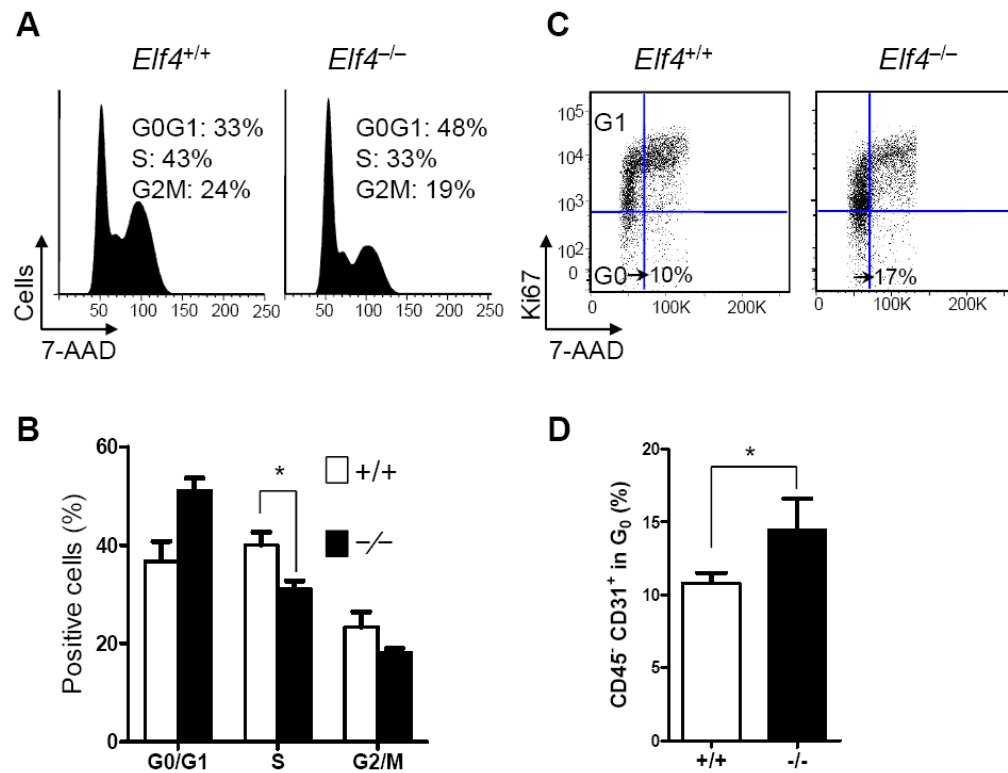
A

Elf4$^{+/+}$

G0G1: 33%
S: 43%
G2M: 24%

Elf4$^{-/-}$

G0G1: 48%
S: 33%
G2M: 19%

Cells

7-AAD

B

Positive cells (%)

□ +/+
■ −/−

*

G0/G1   S   G2/M

C

Elf4$^{+/+}$

G1

Ki67

G0→10%

7-AAD

Elf4$^{-/-}$

→17%

D

CD45$^-$ CD31$^+$ in G$_0$ (%)

*

+/+   -/-

Figure 4. ELF4 inhibits quiescence in bone marrow endothelial cells
(A) DNA content was measured with 7-AAD in CD45$^-$ CD31$^+$ BM cells at steady state. A representative profile is shown. (B) Statistical analysis of DNA content (Two-tailed Student's t-test, n = 3, * P< 0.05). (C) Dual detection of Ki67 and 7-AAD in CD45$^-$ CD31$^+$ BM cells isolated from untreated wild type and *Elf4$^{-/-}$* mice. A representative profile is shown. (D) Statistical analysis of cells in G$_0$ phase of the cell cycle (Two-tailed Student's t-test, n = 3, * P < 0.05).
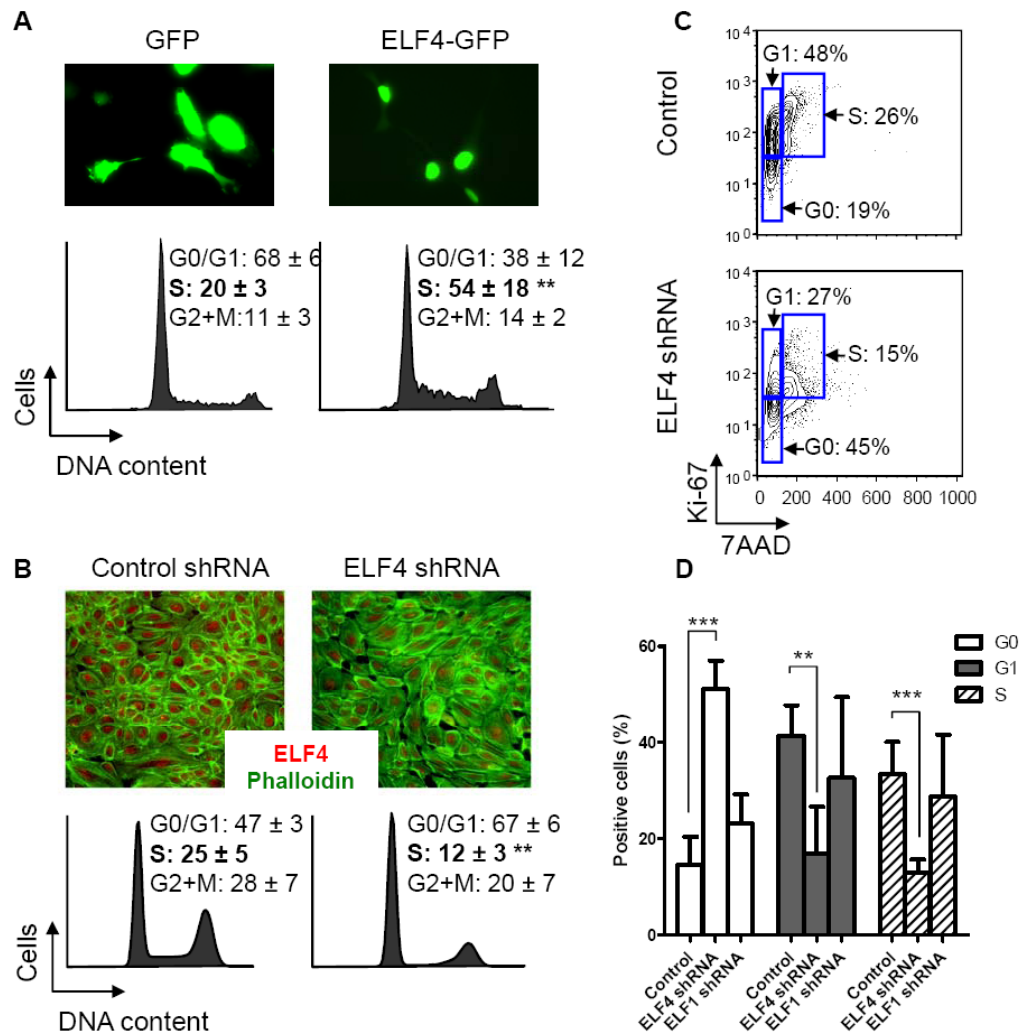
Figure 5. ELF4 promotes cell-cycle entry in HUVEC cells
(A) HUVEC cells expressing the fusion protein ELF4-GFP (top) were used to measure DNA content (bottom). After cell sorting of GFP-positive cells, cell cycle was analyzed using ModFit software. The percentage in each phase of the cell cycle is shown (Two-tailed Student's t-test, ** $P < 0.01$, n = 4). (B) ELF4 expression was silenced in HUVEC cells using shRNA to study its effect on the cell cycle. ELF4 (red) and phalloidin (green) expression in HUVEC cells was performed by immunofluorescence. The percentage in each phase of the cell cycle is shown on the bottom (Two-tailed Student's t-test, ** $P < 0.01$, n = 4). (C) Percentage of quiescent HUVEC cells was analyzed as cells positive for Ki67 with 2n DNA. A representative profile is shown for HUVEC cells transfected with control shRNA or ELF4 shRNA. (D) Statistical analysis of HUVEC cells transfected with control, ELF4 or ELF1 shRNA (Two-tailed Student's t-test, ** $P < 0.01$, *** $P < 0.001$ n = 3)
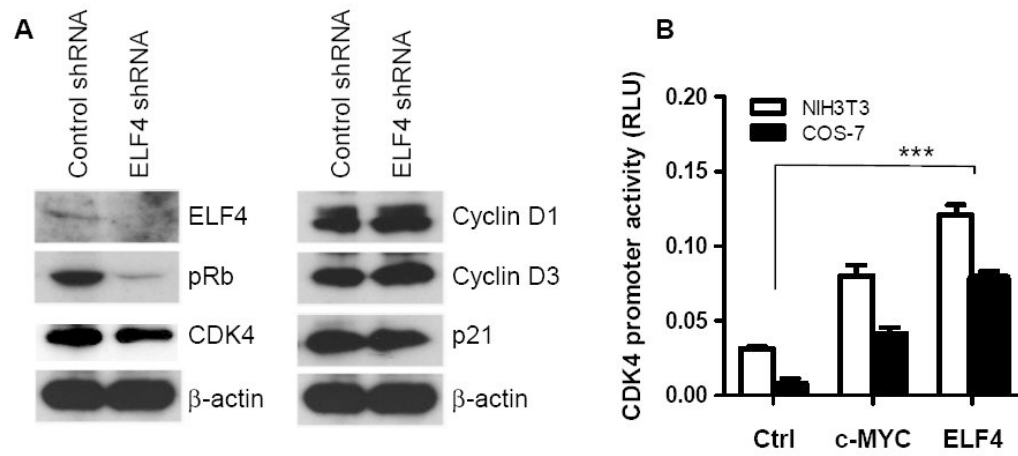
Figure 6. ELF4 activates CDK4 in HUVEC cells
(A) Immunoblot analysis of the expression of ELF4 and cell cycle regulators in HUVEC cells transfected with control or ELF4-specific shRNAs. (B) Capacity of ELF4 to activate the CDK4 gene was determined by promoter reporter assay using COS7 and NIH3T3 cells (Two-tailed Student's t-test, ***$P < 0.001$ compared to control, n = 3).